FINAL REPORT

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PLANT BASED VACCINES

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Final Report

"Plant-based Vaccines" – Project #96000405

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Submitted by VIDO

EXECUTIVE SUMMARY

The overall objective of the project was to develop plant-based vaccines. The reason for proposing this approach was that it was hoped that such an approach would be very economical since we would be able to feed animals the vaccine and induce immunity. This approach would remove the costs of vaccine purification and formulation, especially if we had the vaccine associated with natural oil bodies which act as adjuvants. Secondly, if we could deliver the vaccine orally by feeding, this would reduce the expense of animal handling required for injection and remove the injection site reactions currently estimated to cost $9.25/injection site due to trimming costs. Finally, since the majority of infectious agents enter at the mucosal sites, oral delivery would induce immunity at mucosal sites, thereby help prevent infection rather than just prevent disease as occurs with injected vaccines. Injected vaccines do not induce mucosal immunity due to the compartmentalization of the immune system. Thus, plant-based vaccines appear to be the ideal approach for economical production of effective vaccines for livestock.

In our original submission, we chose BHV-1 as the disease for which we would develop proof of principle. The reason for choosing this disease model is that it is one of the major initiators of bovine respiratory disease, shipping fever, a disease syndrome causing North American livestock producers losses of approximately $1 billion annually. The early efforts made to express BHV-gD in plants was presented in the numerous interim progress reports submitted. Unfortunately, we were unsuccessful in expressing sufficient levels of gD either in canola, our first model, or subsequently in tobacco. The reason for this lack of success was possibly the high GC content of the BHV-1 genes and the need for appropriate glycosylation. As a result, we shifted our focus to other genes. This included VP8 from rotavirus and VP2 from porcine parvovirus as genes for pathogens. These genes were successfully expressed and have been shown to be immunogenic when injected into animals. We are now in the process of growing more plant material and will begin oral vaccination studies.

Other genes we have expressed are involved in immunomodulation. Thus, if we could combine an immunomodulatory agent with a vaccine this should further improve vaccine efficacy. In this regard, we developed and expressed two different immunomodulators – bovine interleukin-6 (BoIL-6) and bovine interleukin-2 (BoIL-2) in plants. Both of these immunomodulators were expressed as cytokine-oleosin fusions in *arabidopsis* and we now have seeds from generation 3 which are currently being analyzed and are being tested for their immunomodulatory activity in cattle.

Finally, we are also expressing BoIFNα as an immunomodulator and have just completed a cattle trial wherein we delivered bovine interferon via either injection or intrapulmonary delivery and showed significant protection from disease just with the immunomodulator alone. This is extremely encouraging since it is the first time that this has been shown to be the case. This provides an opportunity to deliver bovine interferon to cattle as they enter feedlots. This is the time when most animals get exposed to pathogens and suffer the most severe disease. The current practice is to vaccinate upon entry and treat with long-acting antibiotics. Since antibiotics are loosing favor, this would be a novel

approach to protect cattle in the time prior to development of immunity due to vaccination.

The final approach was to develop single-chain recombinant antibodies (scFv) specific for the oleosin protein that could be used to non-covalently link immunogenic proteins to seed oil bodies and determine whether the oil bodies/scFv/immunogenic complex could be used as effective vaccines. We have been successful in developing these scFv's and shown that we could link these to oil bodies and these oil bodies could act as carriers. Preliminary results suggest that this may be an effective way of enhancing immunity.

In summary, we have shown that it may not be possible to use plants as a universal expression system for all antigens, ie. BHV-1 gD, but that expression of some antigens and immune modulators is possible and, more importantly, these plants express antigens that can be biologically active in animals. This project has enhanced collaborations between a company which is committed to developing products in plants, a government research institute, and a university-based research institute committed to improving both the health of animals and the economical liability of the animal vaccine industry in Saskatchewan. A collaboration initiated under this program is continuing and we hope to be successful in developing novel vaccines in the future as a result of this initial funding program.

PROGRESS

We do not propose to re-iterate all of the progress made over the term of the grant since the previous progress reports have been submitted providing all of these details. However, we will summarize the most recent progress. As stated earlier, rotavirus VP8 was chosen as a protein for expression in *arabidopsis*. We have already expressed it previously in tobacco and shown that it was possible to express VP8 in plants and, more importantly, that this VP8 protein could induce immune responses in animals. Thus, we embarked on a program to express VP8 as a fusion protein with *arabidopsis* oleosin sequences for targeting and display on oil bodies.

Since other proteins have been shown to accumulate to higher levels as oleosin fusions compared to the respective soluble form, oil body targeting may serve two purposes. First, it may provide stability and accumulation of VP8 protein in transgenic plants, and second, it will provide the final oil body material displaying VP8 for immunization of animals.

Oil Body Targeted VP8 Protein: Translational Fusion with Oleosin: Two constructs were created for production of VP8 fusions to oleosin. Both N-terminal and C-terminal fusions to oleosin (constructs pSBS4998 and pSBS4999 respectively) were generated to evaluate the compatibility of each topology with stability of VP8 and accumulation of the fusion protein. Each construct was transformed into *arabidopsis* and transgenic lines were recovered. Seed transformed with pSBS4998 was analyzed for accumulation of VP8-oleosin fusion protein (Figure 1).

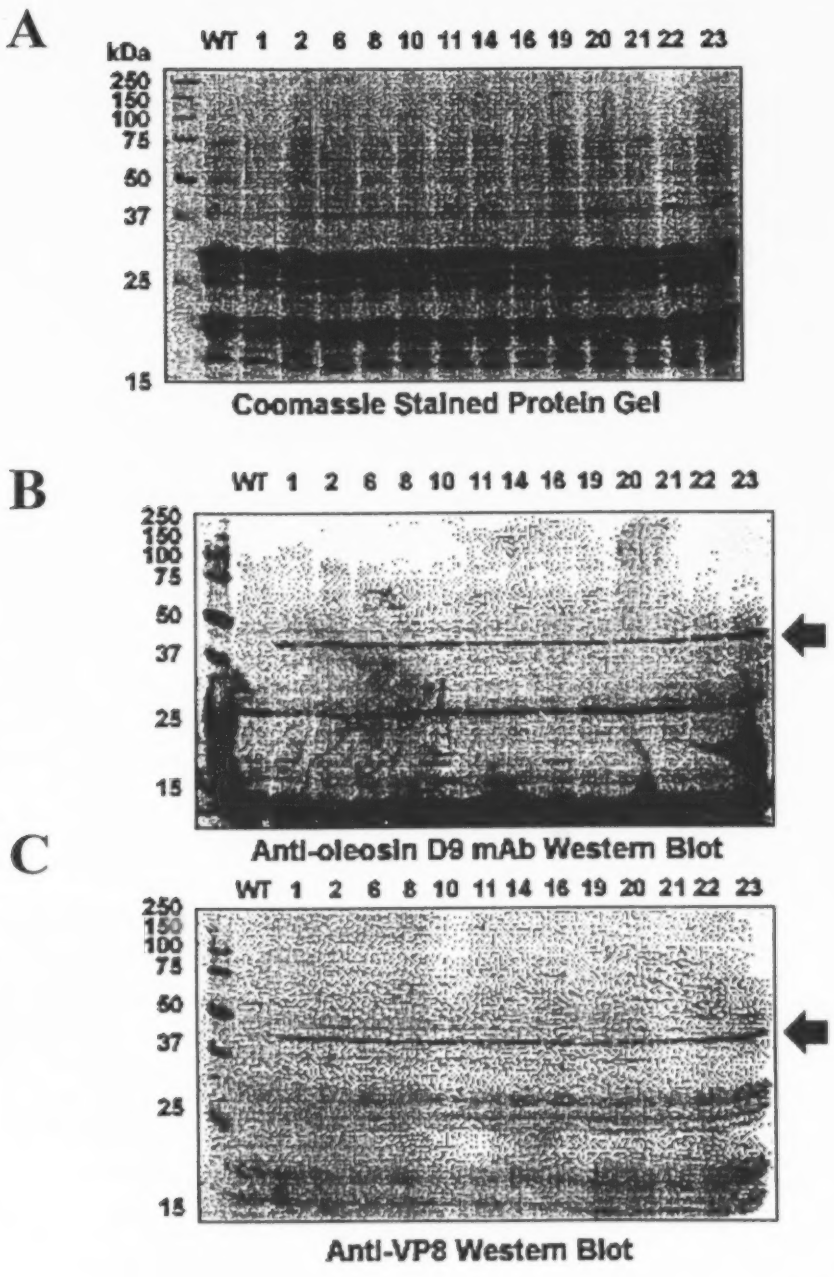

Figure 1. Coomassie stained protein gel (A) and anti-oleosin (B) or anti-VP8 Western blots (C) of total protein extracts from *arabidopsis* seeds. Thirteen lines (1, 2, 6, 8, 10, 11, 14, 16, 19, 20-23) transformed with the VP8-oleosin fusion expression construct (pSBS4998) are compared to wild type (WT) non-transgenic seed. The calculated molecular weight of the VP8-oleosin fusion is 46.1 kDa.

The anti-oleosin (Figure 1B) and anti-VP8 (Figure 1C) Western blots confirm the presence of the fusion protein, and each of the domains respectively, in *arabidopsis* seeds. No apparent degradation of the fusion protein is visible. However, the level of protein production is below the detection limit for visualization on the Coomassie stained gel (Figure 1A). A demonstration of oil body targeting and further quantitation of the VP8 protein level will be necessary to confirm whether or not sufficient antigen can be obtained in an acceptable volume of oil bodies for vaccination into test animals.

The second VP8 fusion construct (pSBS4999; oleosin-VP8) linked with oleosin and may provide higher levels of production and accumulation in seeds. When harvested, the transgenic seed from these lines will be analyzed for oleosin-VP8 expression level. If sufficient protein production is available in one or both of these configuration (pSBS4998 and pSBS4999) then an individual line of each can be grown in the greenhouse and propagated for additional seed. This material will be available for animal testing in a couple of months and extracted oil bodies can be used to determine the antigenic response to VP8.

If the protein accumulation level is less than sufficient then an increase in the efficiency of VP8 fusion protein synthesis may be achieved through re-engineering of the VP8 sequence to create an alternate gene sequence, with equivalent VP8 coding potential, but compatible with plant expression and stability.

Isolation of Recombinant Anti-Oleosin Single Chain Antibodies: A second strategy of linking antigens to oil bodies was to develop single chain antibodies directed against the oleosin. Thus, we could express the antigen linked to the scFv's and then target the oil bodies. This would provide an alternative if the direct fusion with oleosin protein created any problems. Thus, linkage of the antigen could occur without any interference of the oil body formation. Two approaches were used to produce scFv antibodies. These included: a) DNA fragments encoding the immunoglobulin heavy and light chain variable domains were amplified from an anti-oleosin hybridoma cell line (D9) by RT-PCR. The resulting PCR fragments were joined using oligonucleotides encoding a 15 amino acid linker, $(G4S)_3$. The resulting D9 scFv polypeptide (D9ll) was unstable when grown in *E. coli* host strains and we were unable to demonstrate reproducible binding to the oleosin antigen. Upon changing the linker to a five amino acid version (GGGGS) (D9sl) to enhance the formation of scFv dimers, we were able to show reproducible binding to the oleosin antigen. The linker was also modified to a 20 amino acid linker (D9ml) reported by Hennecke et al. (1988) Protein Engineering 11(5):405-440; and b) In the second approach, we used the Recombinant Antibody Phage Display system to isolate anti-oleosin scFv antibodies. A phage display library was constructed and four rounds of affinity selection isolated phages displaying anti-oleosin scFv polypeptides. One of these clones (N766ll) was chosen for further study. This scFv contained the 15 amino acid long linker $(G4S)_3$. The N766 clone was also constructed with the shorter GGGGS five amino acid linker (N766sl). " Figure 2 shows the comparison of the amino acid sequences of N766SL and D9SL.

1. Specificity of the D9sl, N766sl and N766ll for the oleosin protein was confirmed by ELISA assay against several proteins, including purified oleosin protein,

E. coli β-galactosidase (Sigma), *E. coli* DH5α whole cell extracts, purified *Schizosaccharomyces pombe* Cdc4 protein, and bovine serum albumin (Sigma). All three scFv's reacted strongly with purified oleosin protein but did not demonstrate binding to any of the unrelated purified proteins or protein extracts. Western blot analysis demonstrated the recombinant antibodies react exclusively with the oleosin bands in oil body extracts.

2. Stability comparisons demonstrated that the D9sl scFv was stable when stored at –20°C in TBS containing 5mM $MgSO_4$ and 15% glycerol. Both of the N766 clones were stable in either 5 mM $MgSO_4$ pH 6.0 or TBS for several weeks at 4°C, or at –20°C by adding 15% glycerol to the buffer.
3. *E.coli* culture conditions and protein purification protocols were studied to determine the yield of soluble scFv. It was determined that an overnight 30°C culture with 0.4-1 mM IPTG in 2YT plus ampicillin produced satisfactory quantities of scFv both in the culture supernatant and in periplasmic extracts. Gel filtration chromatography demonstrated the scFv proteins form large aggregates (Figure 2). Anti-E-tag affinity chromatography (Pharmacia) of crude N766sl scFv extracts, prior to size separation, enabled isolation of monomeric and dimeric soluble scFv protein.
4. The affinity constant of the monomeric scFv fraction of an N766sl protein preparation was estimated by a modified ELISA method (Beatty et al. 1987. J. Immunol. Methods 100:173-9). The Kaff for this scFv was 3.8×10^{-11}M.
5. In order to determine if the anti-oleosin scFv's will bind to the oleosin protein during the industrial process of purifying the oil bodies from seeds, *Brassica napus* seeds were homogenized using a mortar and pestle in the presence of crude culture supernatants from *E.coli*-expressing secreted scFv's. After centrifugation, the resulting aqueous phase and the oil body fractions were separated from the insoluble seed debris and assayed to determine where the scFv protein localized (Figure 3). The D9sl, N766ll and N766sl all partitioned with the oil bodies while an scFv directed against an unrelated antigen remained in the aqueous phase. This demonstrates that these anti-oleosin scFv's are useful reagents to link immunogenic antigens to the oil bodies and, when expressed in transgenic plants, the scFvs should remain bound to the oil bodies throughout the industrial purification process.

Immunogenicity of Oil Bodies Decorated with Anti-Oleosin scFv/Antigen Fusion Proteins: Our goal with these immunizations was to determine whether an anti-oleosin scFv is able to non-covalently link an antigen to oil bodies and elicit a specific immune response. The first antigen tested was a peptide tag genetically encoded C-terminal to the different versions of the D9 and N766 scFv's. This peptide (E-peptide) is derived from the human osteocalcin precursor protein. A total of 25 mice were immunized with various scFv-Etag fusions captured on the oil bodies. Seven of the 25 mice were included as negative controls and these sera showed no reactivity with the E-tag peptide, as expected. Of the other 18 mice, only two obtained an anti-Etag activity. Both of these were with the N766 long linker version of the scFv. There are several legitimate reasons for such low efficacy (such as tolerance to mouse Fv and poor immunogenicity of the E-peptide): 1) In order to

determine the appropriate dosage, several different dosages were administered. The negative mice may simply not have been exposed to enough antigen, 2) A comparison of reproducibility between the outbred CD1 Swiss strain used in this experiment vs. inbred mouse strains needs to be tested, 3) The E-tag peptide may simply not be very immunogenic, as is the case with many short peptides, and 4) Since the scFv was murine in origin, it may not provide the appropriate T-helper epitopes required for a strong serum response. Indeed, since the murine scFv would be recognized by the mouse immune system as "self", the scFv may inhibit an immune response by inducing tolerance.

FUTURE ENDEAVORS

In order to further study the immunogenicity of antigens linked non-covalently to oil bodies via anti-oleosin scFv's, we will test two globular protein antigens, the Bovine Rotavirus VP8 antigen and the green fluorescent protein (GFP) from the jelly fish. We have three plant lines that express A) GFP as a fusion with oleosin, B) D9 anti-oleosin fused to GFP, and C) D9 anti-oleosin fused to GFP with the KDEL endoplasmic reticulum retension peptide. The benefits of this test system are that GFP is known to elicit immune responses (unlike the E-peptide) and, from preliminary experiments, we know the GFP is displayed on the oil bodies (either by covalent GFP-oleosin fusions or by scFv-GFP non-covalent associations). These plants will demonstrate the development of vaccines is feasible when the scFv/Ag fusion is expressed in plants. Experiments are also underway to express the VP8 antigen in plants with the goal of constructing a plant line that will be useful as a vaccine against a real pathogen.

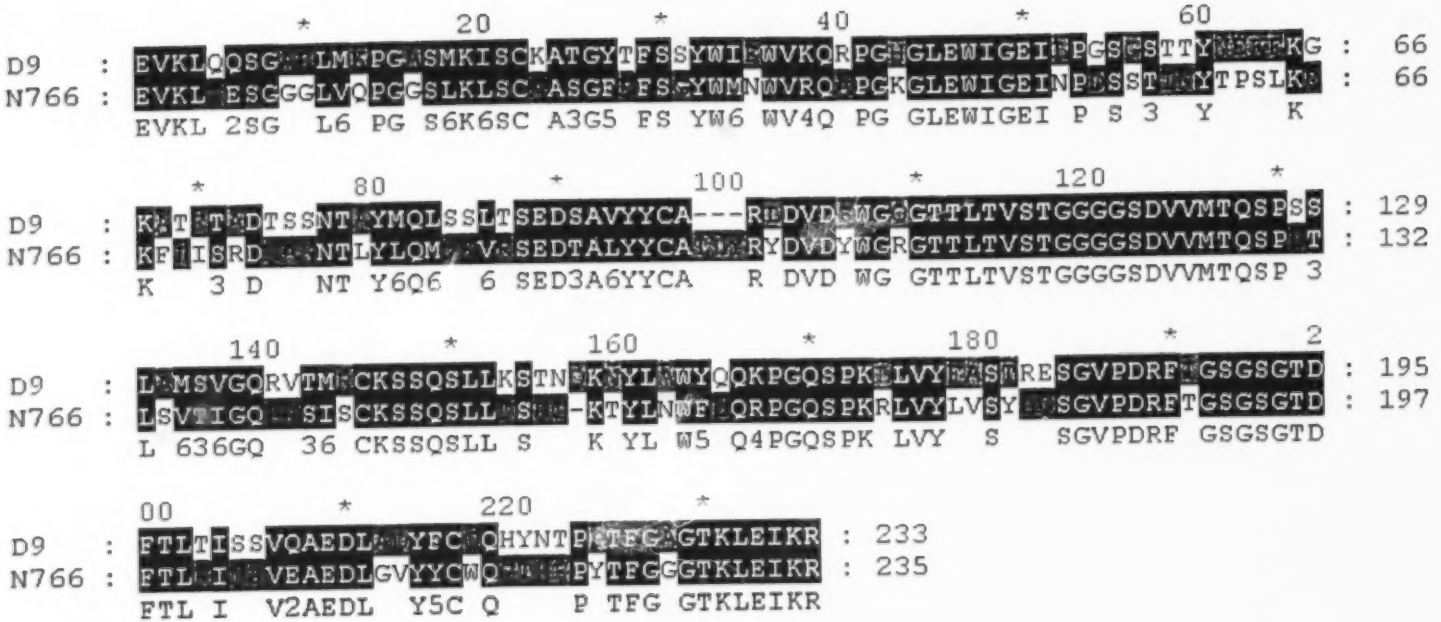

Figure 2. Comparison of the amino acid sequences of D9sl and N766sl.

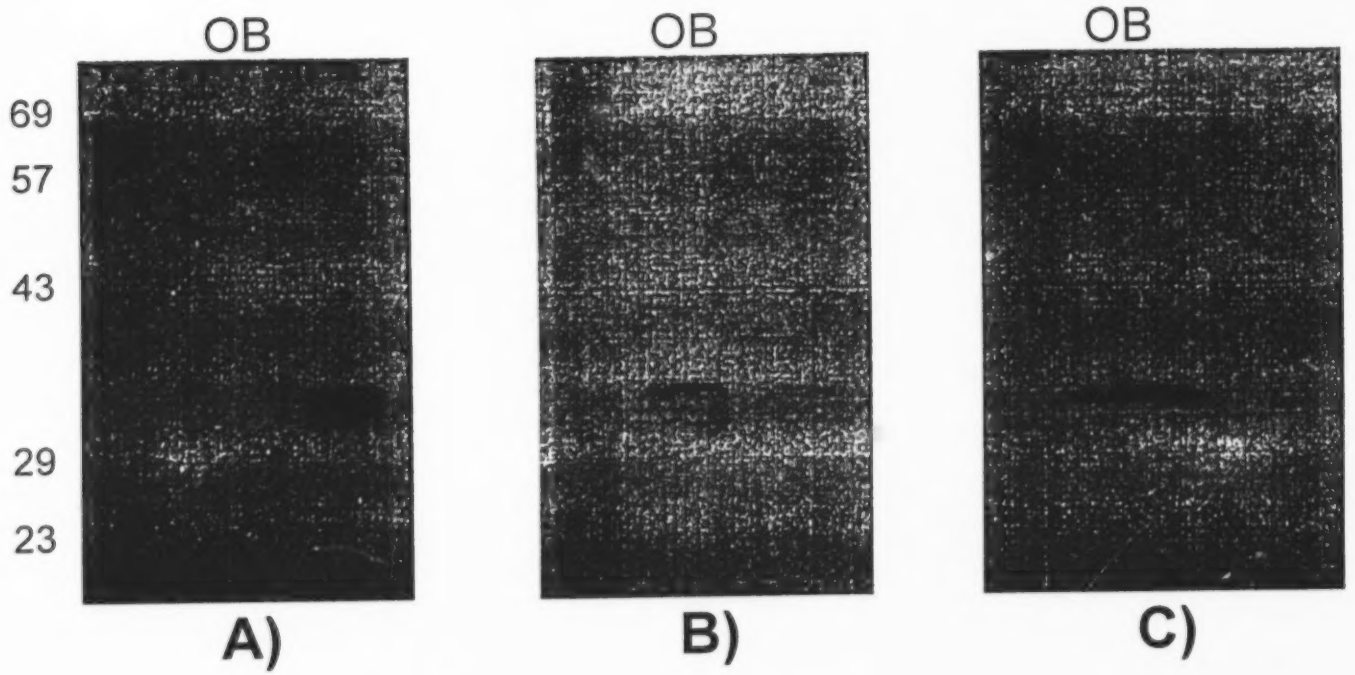

Figure 3. Anti-oleosin scFv's partition with the oil bodies.

Brassica napus seeds were ground in the presence of *E.coli* culture supernatants containing secreted scFv protein. Oil body (OB) fractions and the aqueous undernatant containing unbound scFv (UB) were subjected to SDS-PAGE, transferred to nitrocellulose and tested for the presence of scFv by probing with an anti-E-tag-HRP conjugate. A) anti-APC1 scFv: This non-oleosin specific scFv remains in the aqueous phase and does not associate with the oil body fraction. B) anti-oleosin scFv N766ll: Binds the oil body fraction. C) anti-oleosin scFv D9sl: Binds the oil body fraction.